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Native and Multimeric Vitronectin Exhibit Similar Affinity for Heparin

DIFFERENCES IN HEPARIN BINDING PROPERTIES INDUCED UPON DENATURATION ARE DUE TO SELF-ASSOCIATION INTO A MULTIVALENT FORM*

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For many years, the concept that the heparin-binding sequence is sequestered within vitronectin and exposed upon denaturation of the protein has guided experimental design and interpretation of related structure-function studies on the protein. To evaluate binding of heparin to both native and denatured/renatured vitronectin, methods for monitoring binding in solution have been developed. A fluorescence method based on changes in an extrinsic probe attached to heparin has been used to evaluate heparin binding to native and denatured/renatured vitronectin. This approach indicates that there are not major differences in intrinsic heparin-binding affinities between native and renatured protein and invalidate the currently accepted model for a cryptic heparin-binding sequence in the protein. Denaturation and renaturation of vitronectin under near physiological solution conditions is accompanied invariably by self-association of the protein into a multimeric form (Zhuang, P., Blackburn, M. N., and Peterson, C. B. (1996) *J. Biol. Chem.* 271, 14323–14332), resulting in exposure of multiple heparin-binding sites on the surface of the oligomer. On the basis of the binding data from solution studies and interaction of the native monomer and the denatured multimeric form of vitronectin with a heparin column, along with evaluation of the ionic strength dependence of heparin binding to these vitronectin forms in solution, an alternative model is favored to account for the altered heparin binding properties of vitronectin associated with denaturation of the protein. This model proposes that multivalent interactions between heparin and multimeric vitronectin are responsible for differences in heparin affinity chromatography and ionic strength dependence compared with the native protein.

riety of ligands that are involved in control of diverse physiological processes including coagulation, fibrinolysis, tumor metastasis, the humoral immune response, and cellular migration (reviewed in Refs. 1–6). A partial list of target macromolecules that interact with vitronectin includes heparin, collagen, plasminogen, plasminogen activator inhibitor-1, serine protease inhibitor-protease complexes, and a subclass of integrin receptors on the surface of cells.

Indeed, one of the first molecules shown to interact with vitronectin was heparin (7–11), and this interaction has since been widely investigated (12–16). Much of the early work on vitronectin was devoted to evaluating structural requirements for heparin binding, including tests of the effects of denaturation or proteolysis of vitronectin on heparin binding activity. Binding activity was evaluated from vitronectin interactions with a heparin column, estimated in terms of the salt concentrations required to elute bound vitronectin. From these studies came a most striking observation: vitronectin binding to heparin was enhanced upon treatment of the protein with urea, heat, or acid (9). This phenomenon was exploited by Yatohgo *et al.* (17) to develop an affinity-based purification scheme for vitronectin that invoked chemical denaturation of bulk plasma prior to chromatography on heparin-agarose. As early as 1984, a model emerged in which the heparin-binding region is buried within the native molecule, so that heparin binding is induced when this cryptic binding site is exposed upon denaturation of vitronectin (11, 16). This model has been generally accepted, and more recent work has included attempts to correlate the heparin binding properties of native and denatured/renatured vitronectin with other functions of the protein (12, 18–27). The two forms of the protein, which are sometimes denoted the “non-heparin-binding” (native) and “heparin-binding” (denatured/renatured) species, are characterized in much of the ongoing work in the field.

Many years after the initial work examining effects of denaturation on vitronectin, it was recognized that denaturation is accompanied by self-association of the protein into a multimeric form (28, 29). Multimerization of vitronectin ensues following unfolding of the protein to a structurally altered form that can be detected with a variety of conformationally sensitive monoclonal antibodies (29–31). This partially folded intermediate form has a high propensity to self-associate into multimers that do not readily interconvert with native monomers. The net effect is that multimer formation is essentially irreversible (32). Recently it has been shown that the process of self-association into multimers *versus* refolding to a monomeric form is highly sensitive to ionic strength (31), with multimer formation almost exclusively favored at physiological salt concentrations. From these analyses of denaturation and self-association, it is now clear that the vitronectin species that are commonly clas-

Within the circulation, complicated networks of interactions between proteins and other macromolecules are important for maintaining stasis. An example of the complex interplay that must exist among these biomolecules is provided by the human glycoprotein, vitronectin. Vitronectin interacts with a wide va-

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sified as heparin-binding are, in fact, multimeric forms of the protein.

The native and multimeric forms of the protein are thought to represent the two forms that exist *in vivo*, with most of the circulating pool of vitronectin in a monomeric form, whereas matrix- and other tissue-associated forms appear to be predominantly multimeric. The hypothesis that guided this work is that the two forms of vitronectin exhibit ligand binding properties that differ according to their function and distribution within the body. The goal of this study was to enumerate the energetics of heparin binding to both the native monomer and multimeric vitronectin and to distinguish differences in heparin affinity and differences in numbers of heparin-binding sites on the two forms of vitronectin. In addition to the affinity chromatography approach mentioned above, other methods that have been used previously to evaluate heparin binding to vitronectin have either relied on indirect kinetic methods (14–16, 33) or assays that immobilize one or the other reactant on a plastic surface (16, 28, 29). For a rigorous quantitative analysis of the binding of heparin to both forms of vitronectin, it was essential to develop a solution-based method to monitor the bimolecular interaction. This study provides the first measurements of the affinity and stoichiometry of heparin binding to both forms of the protein using a fluorescently labeled form of heparin to quantify the interactions.

EXPERIMENTAL PROCEDURES

Materials—Vitronectin was purified by a modification of the original procedure of Dahlback and Podack (34), essentially as described by Bittorf *et al.* (28). The molecular weight of the protein is 72,000, and the extinction coefficient is $1.02 \text{ ml} \cdot \text{mg}^{-1} \cdot \text{cm}^{-1}$ (32). Multimeric vitronectin was prepared by treatment of protein in 8 M urea at room temperature for 2 h, with subsequent removal of denaturant by dialysis into 0.1 M sodium phosphate, pH 7.4, containing 0.15 M NaCl and 1 mM EDTA (32). Unbleached, unfractionated heparin was the kind gift of Dr. Michael N. Blackburn (Department of Macromolecular Sciences, SmithKline Beecham, King of Prussia, PA). Human thrombin was provided by Dr. Frank C. Church (Department of Hematology, University of North Carolina, Chapel Hill, NC). The succinimidyl ester of 7-diethylaminocoumarin-3-carboxylic acid was acquired from Molecular Probes. Urea was a product of U. S. Biochemicals. Chromozym-TH was purchased from Boehringer Mannheim. Sephadex G-100 Super Fine was obtained from Pharmacia Biotech Inc. All other chemicals were of reagent grade purity.

Preparation of Coumarin-labeled Heparin—Unbleached heparin was fractionated by molecular weight on two columns in series of Sephadex G-100 Super Fine ($2.5 \times 100 \text{ cm}$ each) linked in series, essentially as described (35). Fractions from the broad elution peak of heparin were combined into separate pools, dialyzed against deionized water, and lyophilized. For labeling with the fluorescent probe, 10 mg of fractionated heparin were dissolved in 1 ml of 0.1 M NaHCO_3 , pH 8.3. 7-Diethylamino-coumarin-3-carboxylic acid, succinimidyl ester (1 mg dissolved in 100 μl of dimethylformamide) was added dropwise to the heparin solution and incubated overnight in the dark at room temperature. Unbound coumarin was removed by exhaustive dialysis into 0.1 M NaHCO_3 , pH 8.3. Labeling stoichiometries were determined spectrophotometrically using an extinction coefficient for coumarin at 432 nm of $57,000 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Heparin concentrations were determined by an azure A dye binding method (36). Molecular weights for size-fractionated, coumarin-labeled heparin samples were determined by sedimentation equilibrium in a Beckman Optima XL-A analytical ultracentrifuge as described previously (35). For ultracentrifugation experiments, heparin samples were dissolved in 2 M NaCl, 20 mM Tris-HCl, pH 7.4, and a partial specific volume of $0.47 \text{ ml} \cdot \text{g}^{-1}$ was used for heparin (37). Absorbance in the ultracentrifugation experiments was monitored at 430 nm, and interference patterns were also recorded at equilibrium. Molecular weights calculated from the absorbance and interference optical patterns were the same within experimental error; this result indicates that the small molecular weight probe was quantitatively bound to the macromolecule, heparin.

Fluorescence Spectroscopy to Measure Heparin Interactions—Binding of heparin to vitronectin was monitored by alterations in fluorescence of the extrinsic coumarin probe that was incorporated into heparin. The change in the fluorescent properties of the coumarin probe upon

vitronectin binding was measured by changes either in quantum yield or polarization. Changes in quantum yield were measured on a Perkin-Elmer LS-50B spectrofluorimeter, using a thermostatted cell holder connected to an external circulating water bath. Small volume aliquots of native vitronectin (at least $20 \text{ mg} \cdot \text{ml}^{-1}$) were added to a microvolume cuvette with a 1-cm path length, containing 150 μl of the labeled heparin sample ($2.0\text{--}30.0 \mu\text{M}$) in 0.02 M Tris-HCl, 20 mM NaCl, pH 7.4. Measurements of the fluorescence emission at 480 nm using an excitation wavelength of 430 nm were recorded in triplicate. The data were mathematically corrected for dilution upon addition of vitronectin. To evaluate binding by changes in polarization of the coumarin probe on heparin, fluorescence anisotropy was measured with an SLM 8000 fluorimeter with film polarizers. Excitation was at 400 nm, and emission was measured at 480 nm utilizing a cut-off filter at 440 nm. Small volume aliquots of vitronectin ($10 \text{ mg} \cdot \text{ml}^{-1}$ stock) were titrated into 2 μM labeled heparin in 20 mM Tris buffer, pH 8.0, containing 20 mM NaCl. The change in fluorescence anisotropy of the coumarin-labeled heparin was monitored as a function of the total native vitronectin concentration.

Binding constants were estimated from heparin titration data using a nonlinear least squares computer fit to the equation:

$$\Delta F = \frac{\Delta F_{\max}}{2R} (R + T + K - ((R + T + K)^2 - 4TR)^{1/2}) \quad (\text{Eq. 1})$$

where ΔF is the change in fluorescence, R is the total concentration of vitronectin-binding sites on heparin, T is the total titrant concentration, and K represents the dissociation constant for heparin-vitronectin interactions (38). Fitting was performed using the Statistical Analysis Software package.

Competitive Binding of Coumarin-labeled and Unlabeled Heparin to Vitronectin—To determine whether the incorporation of an extrinsic probe into heparin affects binding to vitronectin, competitive binding studies using a fixed concentration 3 μM coumarin-labeled heparin in the absence or the presence of 15, 45, or 75 μM unlabeled heparin were performed. Aliquots from a concentrated stock of native vitronectin (at least $20 \text{ mg} \cdot \text{ml}^{-1}$) were added to a microvolume cuvette containing a sample of 150- μl volume. Measurements of the fluorescence emission at 480 nm with an excitation wavelength of 430 nm were recorded. The fluorescence intensity data were mathematically corrected for dilution.

An equation analogous to that described by Bock *et al.* (39, 40) was derived to analyze the competitive vitronectin binding to coumarin-labeled and unlabeled heparin, using the labeled heparin as the probe. The equation can be written as:

$$\frac{[\text{Unlabeled-Hep}]}{[\text{VN}]' - [\text{VN}]} = \frac{K_i}{l \cdot K_d} \left(\frac{1 - \alpha}{\alpha} \right) + \frac{1}{l} \quad (\text{Eq. 2})$$

where the $[\text{Unlabeled-Hep}]$ is the total concentration of unlabeled heparin, $[\text{VN}]' - [\text{VN}]$ is the difference in the total concentration of vitronectin required to achieve the same level of saturation in the presence of the unlabeled heparin and in its absence, K_d and K_i are the dissociation constants for the labeled and unlabeled heparin, respectively, l is the binding stoichiometry, and α is the fractional saturation. The data at values of $\alpha > 0.12$ were analyzed according to the equation.

Fluorescence Analysis of Chemical Denaturation of Vitronectin—Unfolding of vitronectin was induced by urea and monitored by changes in intrinsic protein fluorescence as described (32). Protein fluorescence measurements were made on a Perkin-Elmer LS-50B spectrofluorimeter. Emission spectra were recorded between 300 and 450 nm using an excitation wavelength of 290 nm and a path length of 1 cm. Buffer for the unfolding experiments was 0.1 M sodium phosphate buffer, pH 7.5, containing 0.15 M NaCl, 1.0 mM EDTA and 0.04% (v/v) Tween 20, and the 10 M urea stock solution was made in the same buffer. Unfolding curves were generated from fluorescence measurements on vitronectin incubated overnight in mixtures of the urea stock and buffer to give solutions of intermediate urea concentrations.

Differential Scanning Calorimetry—For differential scanning calorimetry experiments, vitronectin samples were dialyzed overnight against 0.1 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl, 1 mM EDTA. Vitronectin solutions (0.9 mg/ml) in the absence and the presence of added heparin (Sigma, $M_r \sim 6000$) at a 100 μM concentration were scanned at $1^\circ \text{C} \cdot \text{min}^{-1}$ using a high precision differential scanning calorimeter (model DS-93). Samples and reference solutions were properly degassed and carefully loaded into the calorimeter to eliminate artifacts that might arise from spurious bubbles in the solution. Reproducibility of base lines was verified by multiple overnight scans. The analysis of the differential scanning calorimetry data was performed

with software developed by Biocalorimetry Center (Johns Hopkins University, Baltimore, MD) (41). Absolute and excess heat capacities were determined.

Measurement of the Rate of the Heparin-catalyzed Reaction between Thrombin and Antithrombin—Inactivation of thrombin was measured by the ability of antithrombin to inhibit the amidolytic activity of the enzyme essentially as described (38). The rate of inactivation of thrombin by antithrombin plus heparin was monitored continuously by hydrolysis of the thrombin substrate, Chromozym-TH, to product that absorbs at 405 nm. Heparin with an average molecular weight of 6000 (product of Sigma) was used in the kinetic analyses at a final concentration of $1 \mu\text{g}\cdot\text{mL}^{-1}$. The final concentration of chromogenic substrate was 0.19 mM, and thrombin concentration was fixed at 2 nM in the assay. Rates of reaction using a fixed antithrombin concentration of 60 nM were measured at 30 °C in the presence of various amounts of added vitronectin. The standard assay buffer was phosphate-buffered saline (40 mM sodium phosphate, pH 7.4, containing 0.15 M NaCl) containing 0.1% (w/v) polyethylene glycol-8000. Absorbance at 405 nm was recorded on a Perkin-Elmer model Lambda 3B spectrophotometer interfaced with an IBM computer for automated data acquisition. Pseudo-first order rates of reaction were determined by nonlinear least squares analysis using the IGOR software package (Wavemetrics, Oswego, OR).

RESULTS AND DISCUSSION

Because previously published methods for evaluating interactions between vitronectin and heparin have relied either on measurements with one of the species bound to a solid phase or on complicated kinetic experiments with multiple interactions, a primary goal of this work was to develop a method to monitor the binary interaction in solution. Evaluation of changes in fluorescence of vitronectin upon interaction with heparin was an appealing possibility, because the protein contains nine tryptophans at positions 181, 209, 261, 294, 303, 320, 382, 405, and 450 within the 459-amino acid chain. Note that tryptophans 320 and 382 are located in the primary sequence within the general vicinity of the heparin-binding region, which has been localized to amino acids 341–375 (12). However, binding of heparin to vitronectin could not be reliably measured by changes in fluorescence that were attributable to the interaction.¹

Binding of Vitronectin to Heparin Is Detected by Monitoring Changes in the Fluorescence of an Extrinsic Probe on Heparin—Because one of the goals of this research was to compare binding energetics for heparin with both monomeric and multimeric forms of vitronectin, the strategy adopted was to introduce an extrinsic fluorescent probe into heparin that would be sensitive to changes in environment upon binding to either form of the protein. Similar approaches have been previously used successfully to monitor heparin interactions with a variety of proteins, including histidine-proline-rich glycoprotein (38), thrombin (35, 42), antithrombin (35, 43, 44), and fibronectin (45, 46).

Fig. 1 shows binding isotherms in which coumarin-labeled heparin was titrated with native vitronectin. The two panels of the figure illustrate that binding of vitronectin can be measured either by changes in fluorescence intensity (Fig. 1A) or polarization (Fig. 1B) of the coumarin probe. The quantum yield of the coumarin probe is increased upon interaction with vitronectin, and the steady-state anisotropy of the fluor in-

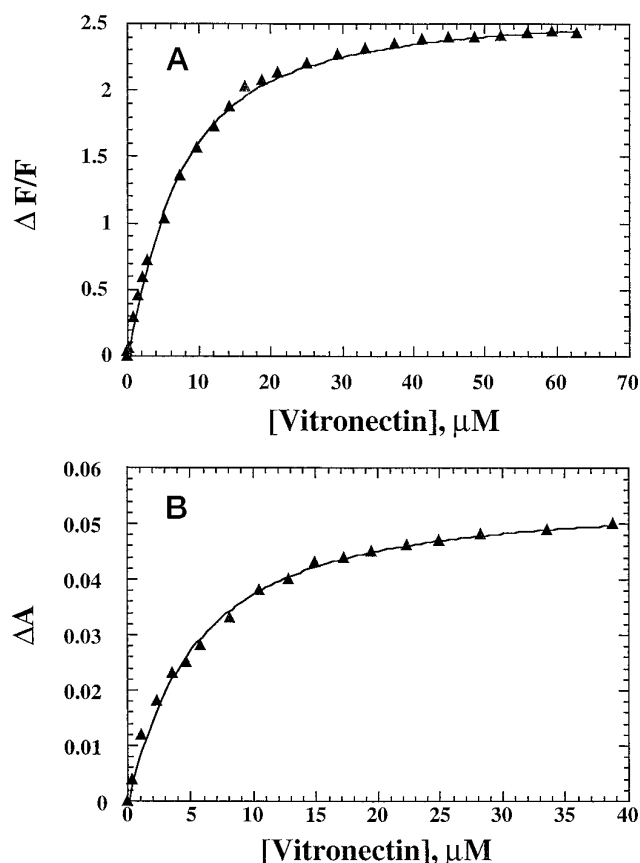


FIG. 1. Vitronectin binding to heparin monitored by changes in fluorescent properties of an extrinsic coumarin probe. A presents a representative binding isotherm for native vitronectin binding to coumarin-labeled heparin, measured by changes in fluorescence intensity at 480 nm, as described under "Experimental Procedures." For this titration, coumarin heparin concentration was fixed at $3.1 \mu\text{M}$, and the fluorescence was monitored upon additions of vitronectin from a stock solution ($20 \text{ mg}\cdot\text{mL}^{-1}$). The line through the data represents the least squares fit, with a calculated dissociation constant of $5.4 \times 10^{-6} \text{ M}$. The heparin sample used for this experiment had an average molecular weight of 9600 determined by analytical ultracentrifugation. ΔF represents the difference between the measured fluorescence at a given concentration of added vitronectin and the initial fluorescence of the probe on heparin, and F represents fluorescence measured for the probe prior to addition of vitronectin. B shows a binding isotherm measured according to changes in fluorescence anisotropy of the coumarin probe attached to heparin. The experiment was conducted essentially as described for A except that changes in polarization of the fluorophore were measured. The heparin sample used for this experiment had an average molecular weight of 6300, and its concentration was fixed at $2 \mu\text{M}$. The line through the data is the best fit from nonlinear least squares analysis, yielding a dissociation constant of $4.2 \times 10^{-6} \text{ M}$.

creases as would be expected upon formation of a molecular complex of larger hydrodynamic volume. The dissociation constants derived from the two equilibrium binding isotherms shown in Fig. 1 are in excellent agreement with that calculated from changes in intensity (Fig. 1A) equal to $5.4 \times 10^{-6} \text{ M}$ and that measured by polarization methods (Fig. 1B) equal to $4.2 \times 10^{-6} \text{ M}$.

Care was taken to establish that the coumarin probe on heparin did not perturb the binding equilibrium with vitronectin by performing rigorous competition studies in which vitronectin-binding isotherms with coumarin-labeled heparin were measured in the presence and the absence of several fixed concentrations of unlabeled heparin (Fig. 2A). These data were used to calculate K_i , the binding constant for unlabeled heparin, and compare it with the binding constant measured for the labeled heparin sample (Fig. 2B). From this analysis, the ratio

¹ A great deal of effort was expended to determine that there is not a reproducible change in the intrinsic fluorescence of the protein upon binding to heparin. Initial results that indicated that vitronectin exhibits a substantial quench in fluorescence upon binding to heparin were misleading; the quench was attributable to a loss of protein in dilute solutions due to adsorption to the cuvette during the course of the experiment. Potential problems stemming from adsorption of vitronectin to the surface of the cuvette were not observed in the experimental protocol that was subsequently pursued, involving titration of a labeled heparin sample with vitronectin. Presumably, a combination of higher protein concentrations used for the titrations and a fixed concentration (in the micromolar range) of the highly charged heparin sample were sufficient to prevent substantial loss of protein during the experiment.

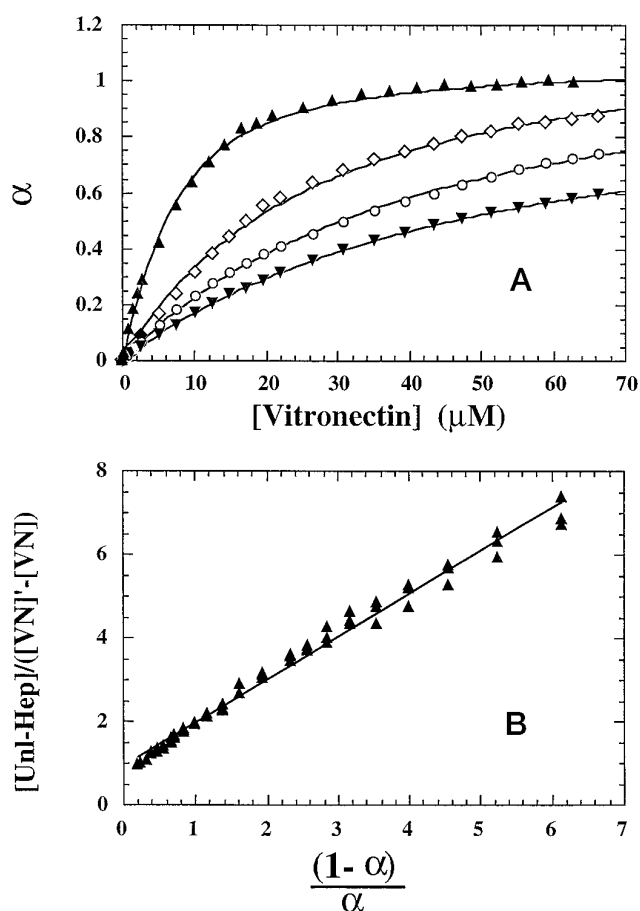


FIG. 2. **Fluorescence titration of coumarin-labeled heparin in the presence and the absence of unlabeled heparin.** A displays titration curves in which a fixed concentration of $3 \mu\text{M}$ labeled heparin was titrated with native vitronectin in the absence of unlabeled heparin (\blacktriangle) or in the presence of unlabeled heparin at a concentration of $15 \mu\text{M}$ (\diamond), $45 \mu\text{M}$ (\circ), or $75 \mu\text{M}$ (\blacktriangledown). The data are expressed in terms of fractional saturation (α). B represents a linearization of all of the data in A exhibiting values of $\alpha > 0.12$ by plotting according to Equation 2. The fitted curves were calculated with a $K_d = 5.37 \mu\text{M}$ obtained from the titration in the absence of unlabeled heparin (shown in A, \blacktriangle). The calculated K_i equals $6.08 \mu\text{M}$, and l , the number of protein binding sites on the ligand, equals 1.1 from this analysis.

between the binding affinities of labeled and unlabeled heparin samples was 1.08, indicating that the coumarin probe itself does not affect the measured binding equilibrium. The competition studies were also important to determine the stoichiometry of interaction between the heparin sample and native vitronectin, which is given from the y intercept of the plot of Fig. 2B. The stoichiometry determined from simultaneous analysis of the three competition curves in Fig. 2 was 1.1 mol of vitronectin bound/mol heparin of molecular weight 9600. It is important to note that size-fractionated heparins were used for all of the experiments described in this paper, because the number of protein binding sites will vary proportionately to the size of the heparin chain.

Binding of Multimeric Vitronectin to Heparin Is Measured by the Fluorescence Assays—Because previous results had indicated that structural changes induced upon denaturation of vitronectin result in apparent higher affinity interactions with heparin, a major objective of this study was to compare binding of heparin with the native protein and vitronectin that had been treated with denaturant. Recent work from this and other laboratories (28, 29, 31, 32) has shown that alteration of vitronectin by chemical or thermal denaturation at physiolog-

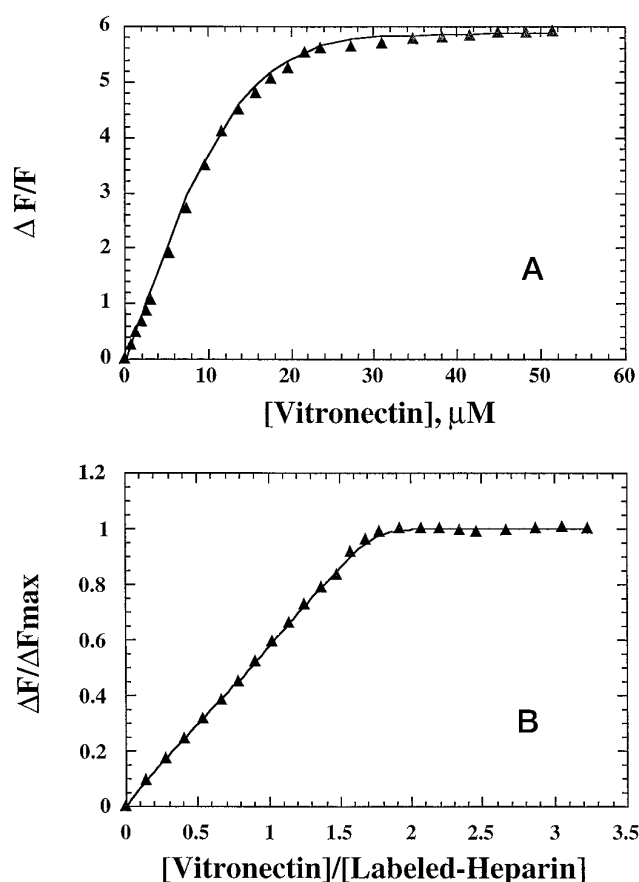


FIG. 3. **Heparin binding to multimeric vitronectin.** A shows the results from titration of the increase in fluorescence of the coumarin probe on labeled heparin as multimeric vitronectin is added. ΔF represents the difference between the measured fluorescence at a given concentration of added vitronectin and the initial fluorescence of the probe on heparin, and F represents fluorescence measured for the probe prior to addition of vitronectin. The heparin sample in this experiment had an average molecular weight of 9600 and was fixed at a concentration of $3.1 \mu\text{M}$. Concentration of vitronectin on the x axis is expressed as the molar concentration of vitronectin protomers within the oligomer. The K_d calculated from the binding isotherm is $5.1 \mu\text{M}$. B, the stoichiometry of multimeric vitronectin binding to the ligand was determined by titrating coumarin-labeled heparin ($M_r \sim 9600$) at a fixed concentration ($30 \mu\text{M}$) well in excess of the measured dissociation constant ($5 \mu\text{M}$) with multimeric vitronectin. The data are expressed relative to an x axis that corresponds to the ratio of total vitronectin added to the cuvette to the fixed heparin concentration in the experiment. Vitronectin concentration is given as total protomers within the multimer. For both panels, the lines through the data represent best fit lines from nonlinear least squares analysis.

ical ionic strengths invariably produces a multimeric form of the protein. The fluorescence methods developed in this work for evaluating binding of heparin to native protein were also useful for evaluating the energetics of interaction of heparin with this multimeric form of vitronectin. Fig. 3 shows results from a binding experiment in which increasing quantities of vitronectin multimer were added to fixed amounts of coumarin-labeled heparin of molecular weight 9600 (same sample used in Figs. 1A and 2 with native vitronectin). Note that the concentration of vitronectin indicated on the x axis corresponds to the concentration of monomeric vitronectin subunits contained within the multimer, *i.e.* the molar concentration of vitronectin monomers (M_r 72,000) rather than the molar concentration of multimeric vitronectin with an average molecular weight equal to 420,000. The dissociation constant for heparin binding to denatured/renatured vitronectin is calculated from the isotherm in Fig. 3A to be $5.1 \times 10^{-6} \text{ M}$, virtually identical to that

TABLE I
Dissociation constants for heparin-vitronectin interactions

Vitronectin form	Buffer	Heparin molecular weights ^a	[Coumarin-heparin] ^b	$K_d \times 10^{6c}$	n^d
			$\times 10^6 M$	M	
Native	20 mM Tris, pH 7.4, 20 mM NaCl	9600	3.14	5.4 ± 0.2	1.1
		14,800	5.05	5.6 ± 0.4	2.1
		19,800	6.30	5.4 ± 0.4	2.7
	20 mM Tris, pH 7.4 Containing 20 mM NaCl Containing 100 mM NaCl Containing 200 mM NaCl	9600	3.14		
				5.4 ± 0.2	1.1
				26.2 ± 1.8	1.1
				53.0 ± 4.0	1.1
	20 mM Tris, 20 mM NaCl pH 6.0 pH 7.0 pH 8.0	9600	3.14		
				6.5 ± 0.3	1.1
				5.6 ± 0.5	1.1
Multimer	20 mM Tris, pH 7.4, 20 mM NaCl	7400	1.85	6.6 ± 0.1	1.0
		9600	3.14	5.1 ± 0.3	1.8
		14,800	5.05	4.4 ± 0.7	3.8
		19,800	6.30	2.8 ± 0.3	5.6
	20 mM Tris, pH 7.4 Containing 20 mM NaCl Containing 100 mM NaCl Containing 300 mM NaCl Containing 400 mM NaCl	9600	3.14		
				5.1 ± 0.3	1.8
				5.5 ± 0.4	1.8
				13.3 ± 1.1	1.8
				40.8 ± 3.6	1.8

^a Molecular weights for unbleached, size-fractionated heparin. The molecular weights were determined by analytical ultracentrifugation as described (35).

^b The fixed concentration of labeled heparin in the experiment. Fixed concentrations of ligand were titrated upon the addition of vitronectin to the sample.

^c Means \pm error from the fit. K_d values were calculated according to Equation 1 under "Experimental Procedures."

^d The parameter (n) represents the binding stoichiometry, defined as the average number of vitronectin monomers (or protomers within the multimer) that bind to a heparin chain of a given molecular weight.

for the native protein. Table I summarizes heparin-binding data, including dissociation constants and stoichiometries of binding, for both the native and multimeric forms of the protein gathered under a variety of experimental conditions.

The distinguishing feature comparing the binding of native and multimeric vitronectin with heparin is the stoichiometry for the protein-heparin interaction. Fig. 3B shows a titration of coumarin-labeled heparin with multimeric vitronectin that was performed using a fixed heparin concentration of 30 μ M, which greatly exceeds the measured K_d . Under these conditions, the titration reaches a clear end point that is a direct measurement of the stoichiometry of the interaction. From this analysis, the stoichiometry of binding equals 1.8 mol of vitronectin protomer/heparin chain. The inference from this analysis is that the heparin-binding site within the multimeric form of the protein spans more than one vitronectin monomer. This interpretation of the data indicates that the binding of heparin to multimeric vitronectin involves more intermolecular interactions than the binding to native vitronectin at a single site on the protein.

It is also instructive to consider the results from the stoichiometry measurement reported in Fig. 3B if protein concentration is expressed in terms of the molar concentration of multimeric vitronectin. This treatment of the data yields a binding stoichiometry of 0.4–0.5 mol of multimeric vitronectin/mol heparin. A stoichiometry of less than 1 is observed in many cases for proteins binding to shorter heparin chains. This result indicates that a binding site large enough to accommodate multimeric vitronectin is not present on all heparins of this size. In contrast, the binding stoichiometry for this heparin sample with native vitronectin is nearly 1:1. This result is as expected, because fewer molecules of larger molecular weight multimer can be bound to heparin chains that are equal in size. If the data in Fig. 3A are expressed in terms of moles of multimeric vitronectin ($M_r \sim 420,000$) with the stoichiometry of approximately 0.5, the best fit of the data yields a dissociation constant of 0.2×10^{-6} M. The tighter apparent binding affinity results from the multiplicity of heparin-binding sites within

multimeric vitronectin.

Thermodynamics of Heparin Binding Are Similar for Native and Multimeric Vitronectin—To further evaluate the contribution of ionic and other types of interactions to the binding of heparin to the two forms of vitronectin, a van't Hoff analysis was pursued in which the temperature dependence of the binding equilibrium was evaluated for native and multimeric protein (Table II). A linear van't Hoff relationship was observed for both forms of vitronectin over the experimentally tested temperature range from 25 to 40 °C. For both the native and multimeric proteins, the binding constant differs 2-fold over the given temperature range. Small differences in the corresponding K_d values at a given temperature that are observed comparing the native and multimeric protein are within experimental error. Because it has been established that different types of intermolecular forces contribute differently to the enthalpic and entropic terms for a given binding equilibrium and because these different types of interactions exhibit different temperature dependences (47), this analysis was pursued to evaluate the type of interactions that account for the free energy of binding heparin to vitronectin. Enthalpic and entropic contributions to the heparin/vitronectin equilibrium, derived from the van't Hoff analysis, are summarized in Table II. These values are compatible with a predominance of ionic forces in the binding interaction, as would be expected from the highly basic nature of the heparin-binding sequence in vitronectin and the polyelectrolytic features of heparin. This analysis demonstrates that the energetics of interaction between heparin and either form of vitronectin are indistinguishable and that the binding interaction is dominated by enthalpic contributions with a smaller contribution from entropic forces. The temperature dependence of the vitronectin-heparin interaction is similar to that for the K_d of the antithrombin-heparin interaction, which varies 1.8-fold in the range from 15 to 37 °C. However, the situation may be contrasted with that for thrombin interactions with heparin, in which there is less of a tem-

TABLE II
Thermodynamics of vitronectin-heparin interactions

Vitronectin form	Experimental		Calculated		
	Temperature °C	$K_d \times 10^6$ M	ΔG (25° C) Kcal·mol ⁻¹	ΔH Kcal·mol ⁻¹	ΔS cal·deg ⁻¹ ·mol ⁻¹
Native	25.0	5.6 ± 0.2	7.2	9.9	9.1
	29.5	7.0 ± 0.4			
	34.0	8.6 ± 0.4			
	38.5	11.8 ± 0.6			
Multimer	25.0	5.1 ± 0.3	7.2	9.5	7.5
	29.5	6.4 ± 0.4			
	34.0	8.3 ± 0.6			
	38.5	10.2 ± 0.8			

perature dependence for the binding equilibrium.²

Heparin Binding Properties of Vitronectin Vary with Heparin Chain Length—The dependence of binding on heparin chain length was examined to determine what effects binding at multiple sites along the chain has on the equilibrium. The data obtained from measurements of native vitronectin binding to three different molecular weight heparin species are shown in Fig. 4A and summarized in Table I. It is apparent from this analysis that the number of sites at which native vitronectin binds on the heparin chain increases with molecular weight, as expected. However, there is not a significant difference in the binding affinities measured with the three different sizes of heparins. This result indicates that vitronectin monomers bind independently at separate sites along the heparin chain.

The question of heparin size effects is especially pertinent for the multimeric form of the protein, in which the heparin-binding site appears to be "shared" between a minimum of two vitronectin protomers within the multimeric protein. One would predict that an interaction that involves multiple binding sites on both the target ligand and the protein would exhibit tighter binding as the number of sites on the ligand increases. Binding isotherms that describe the interaction of multimeric vitronectin with heparins of varying size are shown in Fig. 4B and listed in Table I. The three heparin species are the same that were used in the measurements with native vitronectin in Fig. 4A. In a similar fashion to the native protein, the number of vitronectin protomers bound per heparin chain increases with the molecular size of the heparin. Interestingly, the molar ratio of protomers bound in multimeric vitronectin to the ratio of native vitronectin monomers bound for a given heparin sample is relatively constant (approximately 1.6–2.0) for all of the heparins tested. The consistency of the stoichiometries comparing multimeric and native vitronectin among the heparin series is striking, and it substantiates the argument that the heparin-binding site within multimeric vitronectin encompasses more than one vitronectin polypeptide chain. A further contrast to the independent binding behavior observed with the native protein is the observation that multimeric vitronectin exhibits progressively tighter binding to heparin as the chain length of the polysaccharide increases.

Interaction of Native or Multimeric Vitronectin with Heparin Is Disrupted by Increases in Ionic Strength—The observation that binding constants for heparin with native and denatured/renatured vitronectin are essentially the same was surprising

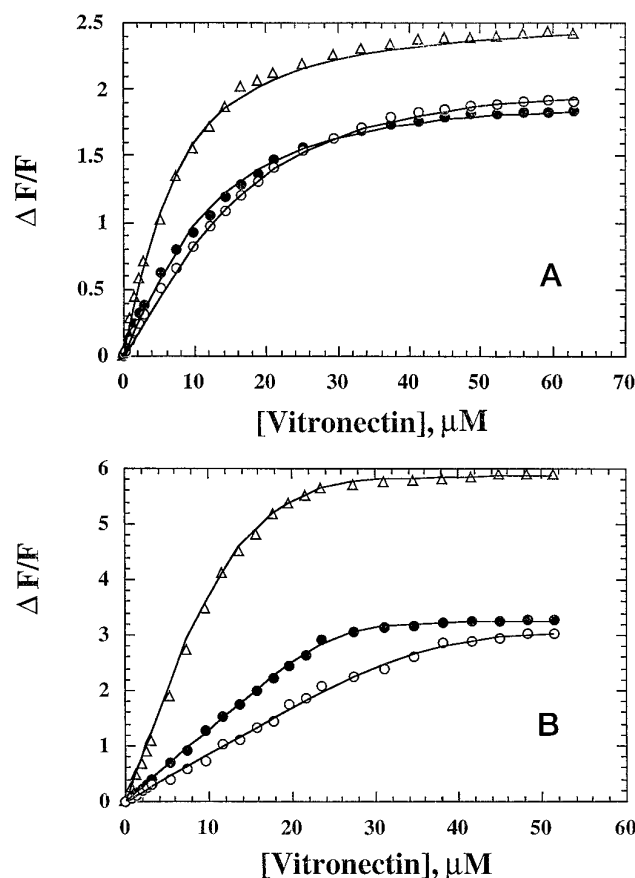


FIG. 4. **Molecular weight dependence of vitronectin binding to heparin.** Vitronectin binding to coumarin-labeled heparins of varying molecular weights was measured by changes in the fluorescence intensity of coumarin probe. Binding isotherms are shown for native vitronectin (A) and multimeric vitronectin (B). Experiments are shown in both panels using heparin with a molecular weight of 9600 at a fixed concentration equal to 3 μ M concentration (Δ), a molecular weight of 14,800 at a fixed concentration equal to 5 μ M (\bullet), and a molecular weight of 19,800 at a fixed 6 μ M concentration (\circ).

in light of the previous work that had indicated tighter binding of the denatured form of vitronectin to a heparin column. To test whether the vitronectin preparations used in this study behaved similarly, interaction of the native and multimeric forms of vitronectin with heparin-Sepharose was evaluated by using a salt gradient elution. The protein is quantitatively bound to the heparin matrix under low ionic strength conditions. Native protein was eluted in a single symmetrical peak with a midpoint near 0.2 M NaCl. However, higher salt concentrations were required to elute multimeric vitronectin from the heparin column, with a midpoint in the elution near 0.4 M NaCl. Thus, similarly to previous findings, the denatured form of vitronectin used in this study bound to a heparin column so that a higher ionic strength was necessary for elution than that required for the native protein.

Ionic strength effects on the binding affinity were measured directly using the coumarin-labeled heparin. Fig. 5A shows titrations of coumarin-heparin with native vitronectin, and Fig. 5B shows titrations with multimeric vitronectin at various salt concentrations. These analyses are summarized in Table I. From these binding isotherms, it is obvious that the native and multimeric forms of vitronectin exhibit different sensitivities to ionic strength variations. Differences are pronounced at near physiological NaCl concentrations, with the native form of vitronectin exhibiting significantly weaker binding ($K_d = \sim 26 \mu$ M) than the multimeric form of the protein ($K_d = \sim 5 \mu$ M) at 100 mM NaCl. It should be noted that the fluorescence of the

² The temperature dependence of the thrombin-heparin interaction was evaluated by Olson *et al.* (51), along with a detailed analysis of the ionic strength and heparin chain length dependence. These studies led to the conclusion that thrombin binding to heparin involves nonspecific electrostatic interactions. This study on vitronectin-heparin interaction does not attempt to distinguish between specific or nonspecific modes of binding. More work with discrete heparin oligosaccharides would be necessary to make such a distinction.

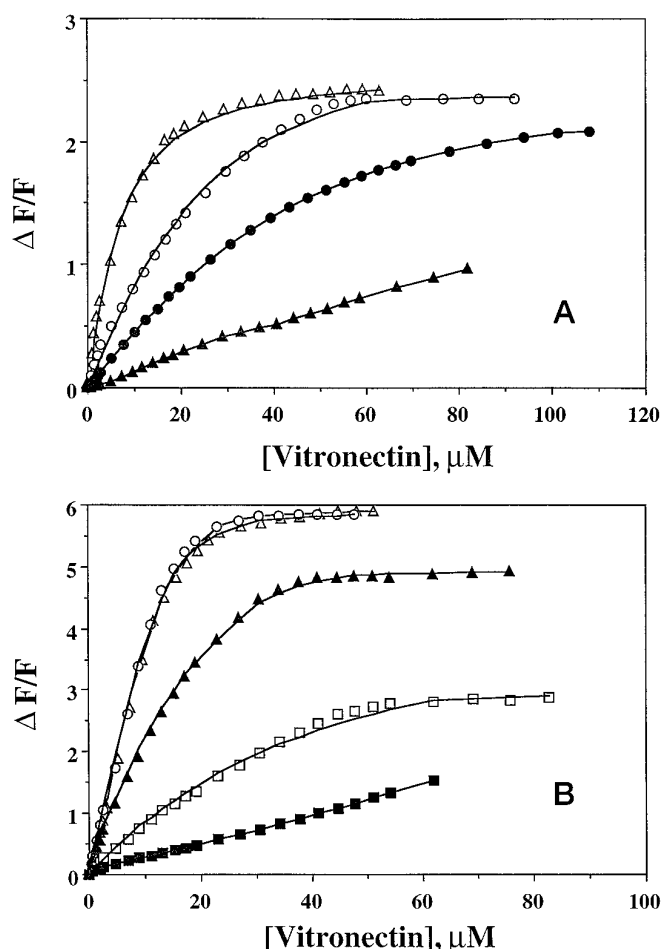


FIG. 5. Ionic strength dependence of vitronectin binding to coumarin-labeled heparin in solution. Titration of vitronectin into a solution of labeled heparin (M_r 9600) was performed at different ionic strengths using changes in the fluorescence of the coumarin probe on heparin to report binding, as described under "Experimental Procedures." The buffer solutions were 20 mM Tris, pH 7.4, containing 20 mM NaCl (Δ), 100 mM NaCl (\circ), 200 mM NaCl (\bullet), 300 mM NaCl (\blacktriangle), 400 mM NaCl (\square), and 500 mM NaCl (\blacksquare). Binding isotherms are shown for native vitronectin (A) and multimeric vitronectin (B). For all of the data sets except the titration of native vitronectin at 300 mM NaCl (A, \blacktriangle) and the titration of multimeric vitronectin at 500 mM NaCl (B, \blacksquare), the line through the data represents the nonlinear least square fit to the equation. The binding data gathered using the highest NaCl concentrations in each of the two panels were sufficiently weak that a titration end point was not reached at the concentrations of protein used in the experiment, so a nonlinear fit to the data was precluded.

coumarin probe on free heparin (*i.e.* that which is not bound to vitronectin) is not altered over the experimental ionic strength range. The direct titrations at various ionic strengths shown in Fig. 5 demonstrate clear differences in binding affinities in the range from 100 to 400 mM NaCl. Higher concentrations of salt are required to disrupt binding of multimeric vitronectin to heparin because of the more extensive binding site that appears to be shared between at least two adjacent vitronectin protomers. More ionic interactions contribute to the binding of heparin to the multimeric form compared with native vitronectin.

The Anticoagulant Activity of Heparin Is Neutralized by Either Native or Multimeric Vitronectin—It was important to determine the effects on heparin anticoagulant activity that resulted from the observed differences in heparin binding properties of the two forms of vitronectin. There has not been a previous comparison of native and multimeric vitronectin in neutralizing heparin using the kinetic assay system. To com-

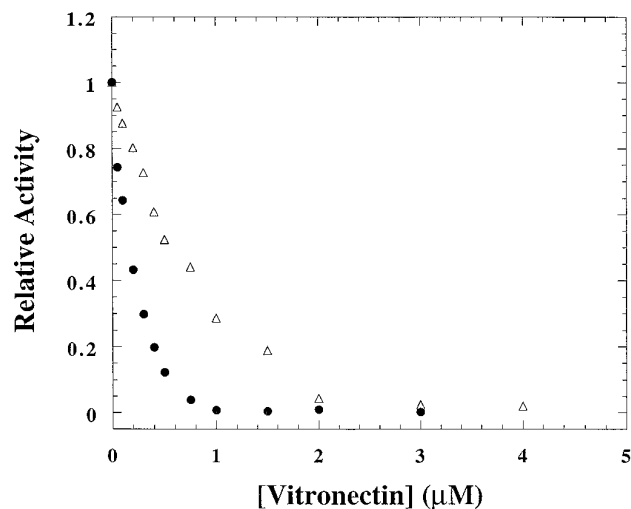


FIG. 6. Neutralization of heparin activity by the two forms of vitronectin. Heparin activity was measured by an increase in the reaction rate of antithrombin inactivation of thrombin. The concentration of active thrombin was monitored continuously over time by hydrolysis of the Chromozym-TH substrate as described under "Experimental Procedures." The effects of vitronectin on the reaction kinetics were measured at varying concentrations and are standardized to the reaction rate of heparin-catalyzed inhibition in the absence of vitronectin. Rates measured at differing concentrations of added native vitronectin are shown in the triangles, and rates measured with varying amounts of added multimeric vitronectin are shown in the circles. Concentrations of multimeric vitronectin are expressed as the concentration of constituent subunits.

pare the consequences of heparin binding by native and multimeric vitronectin on the protease inhibition, reaction kinetics were measured using fixed concentrations of enzyme, inhibitor, and heparin and by varying the concentration of vitronectin. The behavior of native and multimeric vitronectin in this type of assay is compared in Fig. 6. Native vitronectin binds and prevents antithrombin and/or thrombin from binding to heparin in a concentration-dependent fashion. Similar inhibition profiles have been reported previously (15, 16, 48) with a K_i of 0.6 μ M for the heparin-vitronectin interaction estimated from Dixon analysis of kinetic data (15). The results from the present study agree with this estimate, because the concentration of native vitronectin that gives 50% inhibition is almost exactly equal to 0.6 μ M. However, another study by Preissner and colleagues using Factor Xa rather than thrombin as the target protease concluded that the K_d for heparin interaction with native vitronectin was lower, equal to 1×10^{-8} M (16). This calculation was based on attempts to determine concentrations of free and bound heparin from these assay mixtures with multiple components, three of which bind to heparin. The validity of this approach is questionable, and the calculated K_d is 2 orders of magnitude lower than the near micromolar values generated in the current work. Furthermore, the stated K_d is incongruent with the kinetic data from which it is derived, in which effects in the assay are observed using vitronectin concentrations in the micromolar range (15, 16).

For the first time, inhibition of heparin activity by multimeric vitronectin has been measured and compared with the inhibition characteristic of native vitronectin (Fig. 6). The midpoint for inhibition for the multimer is shifted to lower vitronectin concentrations, near 0.2 μ M, compared with the 0.6 μ M value observed for native vitronectin. The rather modest difference in concentrations of the two forms of vitronectin for inhibition of heparin activity reflects the difference in binding affinity of native and multimeric vitronectin at physiological ionic strength. Heparin activity is appreciably inhibited by both forms of vitronectin, an observation that indicates that

heparin binding to either form of the protein may be consequential for the biological activity of heparin.

The ionic strength dependence of the binding interactions observed for native and multimeric vitronectin account for the differences in neutralization of heparin activity in the anti-thrombin-thrombin assay comparing the two forms of vitronectin. The net effect of the stronger binary interaction between heparin and multimeric *versus* monomeric vitronectin at physiological ionic strengths is that lower concentrations of the multimer are required to neutralize the activity of heparin. Heparin-like molecules exist on the lining of the vasculature and are thought to be responsible for maintaining patency of the blood vessels under normal circumstances. Circulating forms of vitronectin are present in micromolar concentrations and are predominantly the monomeric form of the protein, and native vitronectin has the potential to modulate the anticoagulant activity of heparin or heparin-like molecules in much the same way that other proteins that are abundant in the circulation can interfere with heparin activity (38). However, the relative levels of circulating antithrombin (near micromolar), combined with an affinity for heparin that is 2 orders of magnitude tighter than observed with vitronectin, effectively compete for binding of heparin so that its anticoagulant potential *in vivo* is not substantially impaired by vitronectin under normal conditions. In contrast, multimeric forms of vitronectin that are localized to the extracellular matrix and that are released from platelets will be more effective at binding heparin and modulating its anticoagulant potential in the vicinity of thrombi, which are localized to the vessel wall. Thus, the self-association of vitronectin is required to increase the number of heparin-binding sites and effectively compete with antithrombin in circulation for heparin-like molecules at sites of vascular injury or thrombosis.

Heparin Binding to Vitronectin Does Not Stabilize the Protein in Denaturation Experiments—The noteworthy observation that the heparin-binding sites on native and denatured/renatured vitronectin exhibit essentially identical binding constants for heparin, in contradiction to conclusions from most of the earlier work that would predict large differences in binding affinity, prompted a thorough effort to compare and contrast interactions with native and multimeric vitronectin. This laboratory has recently evaluated the denaturation and renaturation/self-association process for vitronectin in detail using spectroscopic and hydrodynamic techniques (31, 32). Reproducible unfolding curves that report changes in protein fluorescence to characterize denaturation of vitronectin with varying concentrations of chemical denaturants have been informative. Shown in Fig. 7A are unfolding curves observed when intrinsic fluorescence of the protein is used to monitor denaturation of vitronectin in the presence and the absence of added heparin. Note that the curves do not differ using the two different experimental conditions tested, although LeChatelier's principle would argue for a difference between the two curves if heparin binds preferentially to the unfolded or denatured form of vitronectin. In that case, the unfolding curve measured in the presence of high heparin concentrations should be shifted to lower concentrations of denaturant, reflecting the preference for heparin binding to the denatured protein and shifting the unfolding equilibrium toward the unfolded form at all concentrations of urea. The absence of any effect of heparin on the unfolding curve supports the conclusion from the binding data and strongly argues that heparin binds with similar affinity to native and denatured vitronectin.

Similar results were obtained in thermal denaturation experiments on native vitronectin. Fig. 7B shows denaturation endotherms for vitronectin measured in the absence and in the

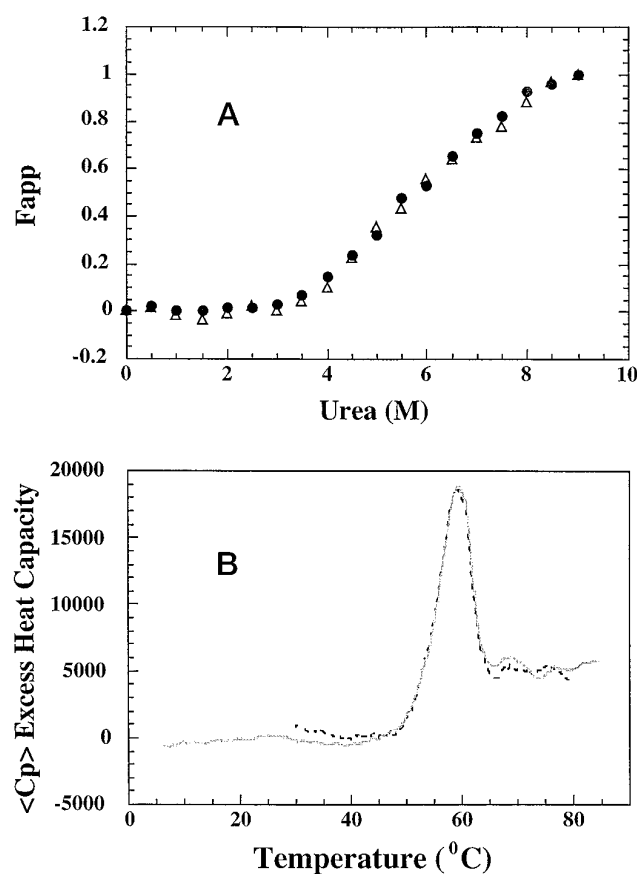


FIG. 7. **Chemical and thermal denaturation of vitronectin measured in the absence and the presence of heparin.** A shows curves that describe the unfolding of native vitronectin in urea, monitored by average tryptophan emission wavelength as described under "Experimental Procedures." Following overnight incubation of vitronectin at each concentration of urea, in the absence (●) or the presence (△) of 20 μ M heparin, emission spectra were recorded from 300 to 450 nm using an excitation wavelength of 290 nm. The vitronectin concentration was 0.5 μ M. The data are expressed as F_{app} , the apparent fraction unfolded. B shows the results of differential scanning calorimetric experiments on vitronectin in the presence and the absence of heparin added in saturating concentrations. Excess heat capacity of vitronectin is plotted on the y axis *versus* temperature on the x axis, measured in the absence (solid line) and the presence (broken line) of 100 mM heparin. Experiments are performed in 100 mM phosphate, 150 mM NaCl, 1 mM EDTA, pH 7.4 buffer. Excess heat capacity values were obtained according to the method described under "Experimental Procedures."

presence of saturating concentrations of heparin. The endotherms are indistinguishable, indicating that heparin neither stabilizes nor destabilizes vitronectin, because preferential binding of heparin to either form should perturb the observed endotherm. Effects of heparin binding on stability would be reflected in changes in the t_m , the midpoint in thermal denaturation of the protein, and perhaps in the enthalpy of denaturation, measured as the area under the endotherm.

An Alternative Model to Account for Differences in Heparin Binding Properties of Native and Multimeric Vitronectin—The model entailing a heparin-binding sequence, which is encrypted in the native fold of vitronectin, as represented schematically in Fig. 8A, has garnered wide support because the initial demonstration that denatured vitronectin binds more efficiently to a heparin matrix than the starting native material (11). Although the early work on the protein clearly pointed to differences in heparin binding properties of the two forms of vitronectin, these differences have not been previously evaluated in a quantitative fashion. Therefore, this work represents the first analysis of the heparin binding properties of the two

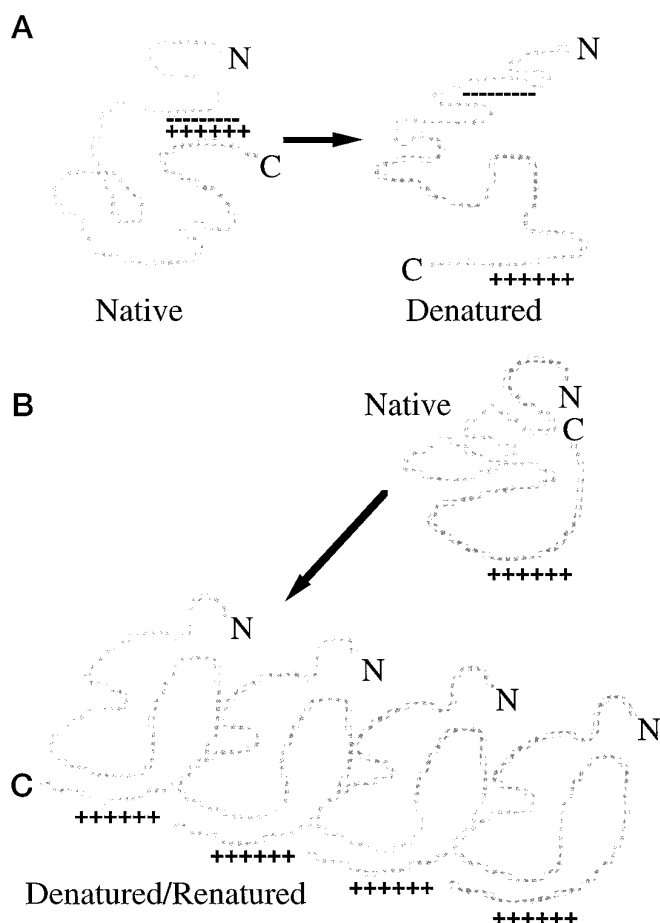


FIG. 8. Potential models to account for the altered heparin binding properties of denatured vitronectin. In A is an adaptation of a model first proposed by Hayashi and co-workers (11), with strong support to follow from Preissner and Muller-Berghaus (16), that invokes a cryptic heparin-binding site in native vitronectin. The heparin binding site is represented by a cluster of positive charges, and intermolecular ionic interactions that are proposed to mask the heparin-binding region in native vitronectin (16) are represented by a string of negative charges. Denaturation of the protein disrupts the proposed intermolecular interactions that shield the region from interaction with heparin in the native form. This model, as originally proposed, does not incorporate multiple binding sites on the multimeric protein produced by denaturation, although such effects do not explicitly affect the basic premise of the model, which is a buried heparin site in native protein. B presents an alternative model for the apparent increase in heparin binding to denatured vitronectin. This model differs drastically from that in A in that the heparin-binding sequence is equally exposed in native and denatured protein. However, denaturation of the protein is accompanied by self-association of vitronectin (28, 29, 31, 32) into a multivalent form that contains proximal heparin-binding sites exposed on its surface. The structural arrangement of monomers within this multimeric form of vitronectin is unknown, so that the regular arrangement of protomers depicted with aligned heparin-binding sites is for illustrative purposes only. According to this scheme, binding to denatured protein is apparently stronger in some binding measurements due to the increased number of heparin-binding sites. N, N terminus; C, C terminus.

forms of vitronectin using solution-based methods. Previously, distinctions in the concentration of salt required to elute the native and multimeric forms of vitronectin from a heparin column have been noted (9, 11, 13), but these data cannot be interpreted in a straightforward manner in terms of binding affinity. Furthermore, immobilization of one or the other interacting species on a solid phase (*e.g.* matrix or plastic surface) in many cases perturbs the binding equilibrium so that it is not reflective of the situation in solution. Although this has not been considered for heparin-vitronectin interactions, the problem has surfaced in evaluating vitronectin interactions with

certain other target ligands (49).

For these reasons, it was imperative that a solution-based method for measuring heparin-vitronectin interactions be developed. Although the solution biochemistry clearly establishes similar binding behavior for native and multimeric vitronectin, there is obviously a difference in effective heparin binding "affinity" for immobilized heparin and ionic strength requirements for the interaction. How can these two results be reconciled? To visualize the differences between the measured equilibria comparing monomeric and multimeric vitronectin, it is helpful to consult the model presented in Fig. 1B. Following denaturation and renaturation, vitronectin associates with itself to form a noncovalently linked multimeric protein. Exposed on the surface of the protein are multiple binding sites for the target ligand, heparin. The ionic strength and molecular weight dependence of the binding interaction appears to be the result of a more extensive binding site within the multimer that incorporates features from individual heparin binding sites on adjacent vitronectin protomers comprising the multimer. Juxtaposition of these multiple binding sites increases their local concentration so that rebinding of the ligand following dissociation is favored. Because it is clear that there is only a single heparin-binding site per vitronectin in its native, monomeric form, the dissociation and reassociation of heparin from native protein is not influenced in this way. This situation described for heparin interactions with multimeric vitronectin is analogous in many respects to the paradigm of avidity effects in binding of antigens to bivalent (or multivalent) antibodies, in which effective binding of antigen to the antibody molecule is much tighter than the measured binding affinity of the separate antigen-combining sites.

Most significantly, the model that is supported by this work (Fig. 1B) does not involve burial of the heparin-binding site within the native structure of the protein. Masking of the heparin site within vitronectin has been widely accepted, despite the fact that the kinetic measurements that were used to initially demonstrate neutralization of heparin by vitronectin were performed using native protein and were used to calculate K_i values representing appreciable affinity for heparin (14, 16, 33). Specific interactions with a relatively acidic region of vitronectin near the N terminus have been proposed previously to mask the heparin binding site in the native protein, with little evidence to support this notion aside from charge considerations (16). As discussed above, the effectiveness of native vitronectin in modulating the anticoagulant activity of heparin certainly discourages application of a model in which the binding site is masked. Furthermore, the heparin-binding region of vitronectin has recently been shown in this laboratory to be susceptible to chemical modification with an arginine-specific fluorescent probe (52), a result that also indicates that this region of the protein is surface-exposed and accessible. It is clear from this work that the heparin binding sites within native and multimeric vitronectin are similarly exposed so that intramolecular interactions involving the acidic region of vitronectin cannot be envisioned to bury the heparin-binding sequence. The two types of vitronectin should no longer be referred to as heparin-binding or non-heparin binding; they are more appropriately identified as native (or monomeric) and multivalent (or multimeric) forms of vitronectin.

The physiological mechanisms that convert vitronectin from a native to a structurally altered multimeric form with an increased local concentration of binding sites for heparin remain elusive. Heparin binding induces conformational changes in native vitronectin that can be detected with monoclonal antibodies (24, 50); although such conformational changes are generally perceived as prerequisites for the self-association of

vitronectin leading to multimeric forms, the binding of heparin in itself is not sufficient to induce multimerization of the protein (28, 31). Other studies using conformationally sensitive monoclonal antibodies have indicated that most noncirculating forms of vitronectin exist in the conformationally altered form. From this information, it has been inferred that the form of vitronectin found in the extracellular matrix is multimeric in composition. Self-association to a multimeric form in the extracellular matrix may be advantageous to enhance binding of other macromolecules known to associate with vitronectin, including plasminogen activator inhibitor-1, collagen, and heparin-like molecules. For other macromolecular interactions altered upon denaturation of vitronectin, it will be interesting to consider whether perturbations are due to alterations in intrinsic affinity or clustering of binding sites on the multimer and thus to clarify the importance of the observed conformational lability of vitronectin in regulation.

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